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RNA intermediates in potato spindle tuber viroid replication

(complementary RNA/replication model)

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ABSTRACT **Two double-stranded RNA intermediates of viroid replication have been isolated from potato spindle tuber viroid (PSTV)-infected tomato tissue and characterized by polyacrylamide gel electrophoresis and DNA·RNA hybridization techniques. These replicative intermediates contain monomeric circular or linear PSTV strands complexed with a multimeric complementary RNA strand. Synchronous synthesis of single-stranded PSTV is accompanied by a simultaneous marked increase in double-stranded PSTV RNA; thus, *in vivo* precursors of the characterized double-stranded PSTV RNAs seem to be involved in PSTV replication. A "rolling circle" model for viroid replication on a circular PSTV template can accommodate the double-stranded PSTV RNA species characterized.**

Viroids are the smallest autonomously replicating pathogenic agents known, consisting solely of a small unencapsidated single-stranded RNA (for review, see ref. 1). The complete 359-nucleotide sequence of potato spindle tuber viroid (PSTV) is known, and detailed models of the secondary and tertiary structures have been presented (for review, see ref. 2). Our understanding of the precise mechanism of viroid replication is not as advanced, however, as is our structural knowledge.

Contrary to an earlier report (3), DNA sequences complementary to PSTV cannot be detected in infected tissue (4–6); thus, viroid replication appears to involve RNA-directed RNA synthesis. There is convincing evidence for the presence of RNA complementary to citrus exocortis viroid in viroid-infected, but not in healthy, tissue (7, 8). Owens and Cress (9) have shown that RNA complementary to PSTV (cPSTV) can be isolated from infected tomato tissue and that ribonuclease-treated RNA preparations contain cPSTV that is the same size as PSTV. Most of this complementary RNA appears to occur as ribonuclease-resistant duplexes with PSTV. *In vitro* experiments suggest that the enzyme responsible for viroid replication is host cell DNA-dependent RNA polymerase II (10).

At least two types of virus-related RNA species, the double-stranded replicative form (RF) and the partially double-stranded replicative intermediate (RI), may be involved in the replication of small plant RNA viruses (for review, see ref. 11). Although the enzymes catalyzing viroid and RNA plant virus replication are apparently different, one might expect viroid replication to involve analogous double-stranded complexes of viroid and viroid-complementary RNA. In an extension of our previous studies of cPSTV, we have indeed detected such double-stranded RNA intermediates in PSTV replication. Characterization of these intermediates showed some striking differences from the RF and RI species typical of RNA plant virus replication.

MATERIALS AND METHODS

Isolation and LiCl Fractionation of Viroid RNA. A severe strain of PSTV (gift from M. Zaitlin, Cornell University) was propagated in tomato (*Lycopersicon esculentum* Mill., cv. Rutgers), and total cellular RNA was prepared from frozen leaf tissue by direct phenol extraction (12). Polysaccharides were removed from the pooled aqueous phases by 2-methoxyethanol extraction and DNA was removed by treatment with DNase. LiCl fractionation of total RNA, if desired, was accomplished by adding an equal volume of 4 M LiCl to solutions containing total RNA at 2 mg/ml. Yields of RNA were ≈750 μg/g of leaf tissue (≈200 μg of LiCl-soluble and 550 μg of LiCl-insoluble RNA).

Isolation and Pancreatic Ribonuclease Sensitivity of Double-Stranded PSTV RNA. Single-stranded (ss) and double-stranded (ds) PSTV RNAs were separated by CF-11-cellulose chromatography at room temperature (13, 14). RNA samples were dissolved in 50% ethanol/STE buffer (100 mM NaCl/50 mM Tris·HCl/1 mM EDTA, pH 7.0) and applied to the column (1 mg of RNA/5 ml of bed volume). The column was washed with 50% ethanol/STE buffer and the ss RNA and ds RNA fractions were eluted with 17.5% ethanol/STE buffer and H_2O, respectively. Yeast tRNA (10 μg/ml) and 0.2 M KOAc were added as required before recovery of the RNA fractions by ethanol precipitation.

RNA samples (≤2 mg/ml) in 0.36 M NaCl/40 mM Na cacodylate/2 mM EDTA, pH 7.0, were incubated with pancreatic RNase (20 μg/ml; Calbiochem; five-times crystallized) for 15 min at 37°C to degrade ss RNA. RNase digestion was stopped by addition of 0.4% $NaDodSO_4$ and phenol/$CHCl_3$ extraction. After ether extraction of the residual phenol and addition of diethyl pyrocarbonate to 0.1%, RNase-resistant RNA was recovered by ethanol precipitation in the presence of yeast tRNA carrier.

Gel Electrophoresis, Electrophoretic Transfer to Diazobenzyloxymethyl (DBM)-Paper, and Hybridization Analysis of PSTV-Related RNA. Fractionation of RNA samples by electrophoresis in 5% acrylamide/0.125% *N,N'*-methylenebisacrylamide gels containing Tris borate/EDTA/8 M urea at 55°C has been described (5). Unless the RNA samples are heated at 100°C before electrophoresis, ds RNA is not denatured. RNA was extracted from preparative gels by the procedure of Maxam and Gilbert (15).

Fractionated RNA samples were transferred to DBM-paper by electrophoresis (16–18). The ethidium bromide-stained gel was incubated at room temperature with 250 ml of 50 mM NaOH (40 min), 250 ml of 0.15 M NaOAc/0.85 M HOAc (10 min), and 250 ml of 17 mM NaOAc/94 mM HOAc (10 min). Electrophoretic transfer (4 hr at 10 V/cm in the presence of 17 mM NaOAc/94 mM HOAc) of the denatured and fragmented RNAs to DBM-paper was virtually quantitative.

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Abbreviations: PSTV, potato spindle tuber viroid; cPSTV, RNA complementary to PSTV; $PSTV_C$ and $PSTV_L$, covalently closed circular and linear PSTV molecules, respectively; ss, single stranded; ds, double stranded; RF, replicative form; RI, replicative intermediate; DBM, diazobenzyloxymethyl.

A single buffer mixture (40% formamide/0.18 M NaCl/20 mM Na cacodylate/1 mM EDTA/0.1% $NaDodSO_4$, pH 7.0 containing yeast tRNA at 400 μg/ml) was used for the prehybridization and hybridization reactions. Prehybridization (16 hr at 42°C with 1% glycine); hybridization (24 hr at 55°C with 10% dextran sulfate), and subsequent washings were performed essentially as described (16). Five milliliters of buffer mixture containing 5×10^5 cpm of [^{32}P]DNA probe was used for a 7 × 10 cm DBM-paper. Autoradiography with Kodak X-Omat film and Dupont Cronex Lightning Plus intensifying screens was carried out at −70°C.

Preparation of Specific Hybridization Probes. The molecular cloning of PSTV cDNA has been described (9), and construction of clones containing the complete 359-nucleotide sequence of PSTV by ligation of specific fragments from overlapping PSTV cDNA clones is presented elsewhere (19). These clones contain full-length ds PSTV cDNA inserted into the *Hin*dIII site of pBR322 via synthetic oligonucleotide linkers; digestion with *Hae* III releases a 359-base pair PSTV-specific fragment.

Hybridization probes specific for either PSTV or cPSTV were prepared by labeling the 5′ termini of *Bam*HI-cleaved plasmid DNA with [γ-^{32}P]ATP (3000 Ci/mmol; Amersham; 1 Ci = 3.7×10^{10} becquerels) and polynucleotide kinase (Boehringer Mannheim), digestion of the [^{32}P]DNA with *Hae* III, and electrophoretic purification of the two PSTV-specific fragments (15). The 300- and 59-base pair fragments provide specific probes for nucleotides 147–87 of PSTV and 88–146 of cPSTV, respectively. The initial specific activity of these strand-specific probes was $1–8 \times 10^7$ cpm/μg.

All operations involving recombinant DNA were performed in accordance with the current National Institutes of Health guidelines for recombinant DNA research.

RESULTS

Relative Amounts of ss and ds PSTV RNA Species. Little PSTV or cPSTV was present in the 2 M LiCl-insoluble fraction; recovery of PSTV-related RNAs in the 2 M LiCl-soluble fraction containing primarily low molecular weight RNA and ds nucleic acids was virtually quantitative (Fig. 1). Circular ($PSTV_C$) and linear ($PSTV_L$) forms of ss PSTV were abundant, but only traces of the corresponding full-length ss cPSTV molecules ($cPSTV_C$ and $cPSTV_L$) could be detected. A more abundant cPSTV species, migrating more rapidly than the $PSTV_L$ marker, appears to be a specific single-stranded fragment of cPSTV (data not shown).

Two or more RNA species migrating more slowly than the $PSTV_C$ and $PSTV_L$ markers and apparently containing both PSTV and cPSTV are also visible. These slowly migrating zones could contain ds RF and RI molecules characteristic of RNA-directed RNA synthesis, but other alternatives—aggregates or multimeric forms of ss PSTV and cPSTV—are possible. Incubation with pancreatic RNase in the presence of high salt was used to distinguish among these alternatives.

Identification and Characterization of ds PSTV RNAs. Two discrete zones of PSTV-related RNA survived pancreatic RNase digestion of low molecular weight RNA in the presence of 0.36 M NaCl (Fig. 2); the mobility of the more rapidly migrating zone (lanes 1 and 5) was slightly less than that of ds cucumber mosaic virus-associated RNA 5, which contains 335 base pairs (20). The two species were extracted from a preparative gel and analyzed by electrophoresis after denaturation. The more slowly migrating zone contained both $PSTV_C$ and $PSTV_L$ but only $cPSTV_L$, while the more rapidly migrating species contained only $PSTV_L$ and $cPSTV_L$. The stringent RNase digestion protocol has also randomly nicked a portion of the PSTV and cPSTV strands.

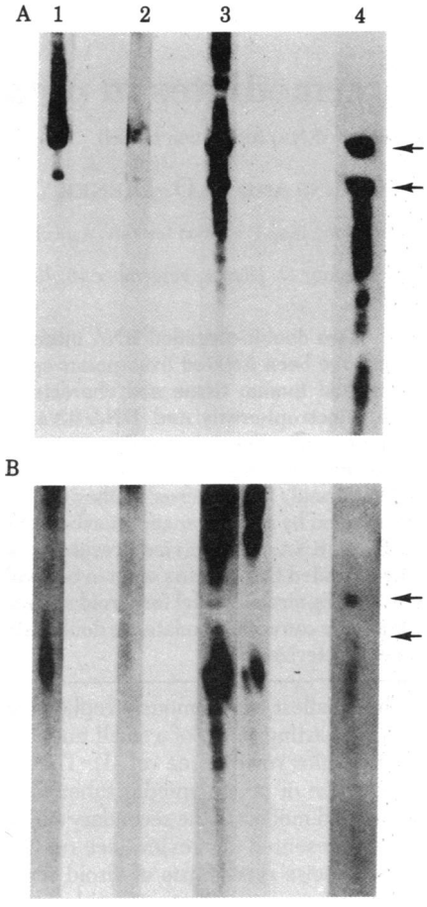

FIG. 1. Fractionation of PSTV (*A*) and cPSTV (*B*) species in 2 M LiCl. Twenty micrograms of RNA from each fraction [total (lane 1), 2 M LiCl-insoluble (lane 2), and 2 M LiCl-soluble (lane 3)] and 80 ng of PSTV purified by one cycle of gel electrophoresis under nondenaturing conditions (lane 4) were subjected to electrophoresis, transferred to DBM-paper, and hybridized with the appropriate [^{32}P]DNA probe. Autoradiographic exposures for the cPSTV analyses were longer than those for PSTV analyses (12.5-fold longer for the purified PSTV and 7-fold longer for the other RNA fractions). Equal length exposures of the 2 M LiCl-soluble RNA fraction are included for comparison. Arrows, $PSTV_C$ (upper) and $PSTV_L$ (lower) markers.

Further characterization of ds PSTV RNAs required a method of removing the ss PSTV and cPSTV from 2 M LiCl-soluble RNA without concomitant alteration of the ds RNAs. Preliminary experiments showed that chromatography on CF-11-cellulose and preparative electrophoresis could resolve two discrete ds PSTV RNA zones; an analysis of the sizes of the PSTV and cPSTV components is shown in Fig. 3. Although denaturation released only $PSTV_C$ and $PSTV_L$ (as well as fragments), the cPSTV molecules that were released migrated more slowly than the $PSTV_L$ marker (lanes 2 and 6). These cPSTV molecules appear to contain >359 nucleotides. Comparison with cucumber mosaic virus ss RNAs run in parallel suggested that the cPSTV strands were linear molecules of various lengths with molecular weights of 130,000 to at least 260,000. cPSTV released from the more slowly migrating ds PSTV RNA was distinctly longer than cPSTV released from the more rapidly migrating ds PSTV RNA (compare lanes 2 and 6).

Pancreatic RNase digestion suggests that the RNA species in these two zones are structurally related. The more slowly migrating zone was converted to the more rapidly migrating zone and a RNA·RNA duplex containing $PSTV_L$ and $cPSTV_L$ (compare lanes 1, 3, and 5 with Fig. 2). Although the size of the

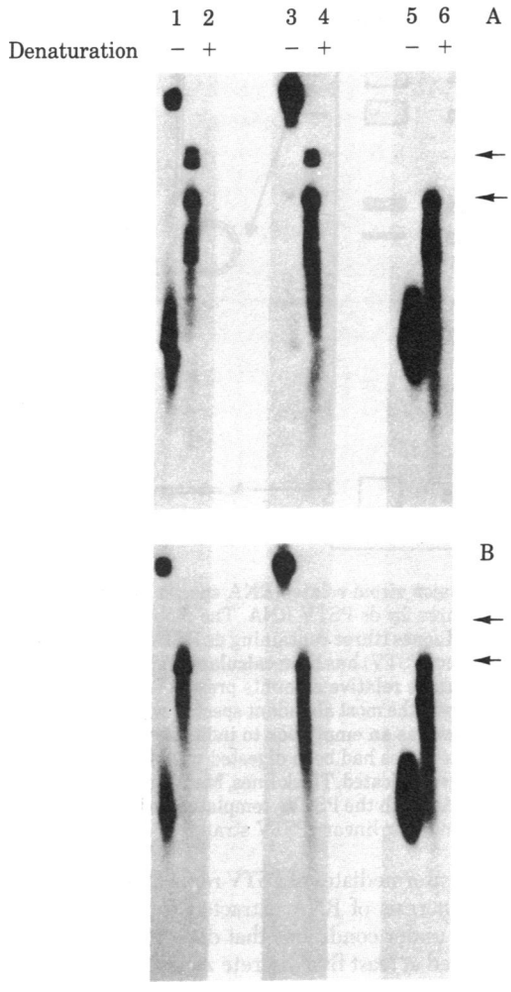

FIG. 2. Characterization of RNase-resistant ds PSTV RNA species. RNA soluble in 2 M LiCl (500 μg) was incubated with pancreatic RNase and recovered by phenol extraction and ethanol precipitation. RNA equivalent to 50 μg of 2 M LiCl-soluble RNA was retained for analysis, while the remainder was fractionated by preparative gel electrophoresis. Each sample lane contains RNA equivalent to 12.5 μg of 2 M LiCl-soluble RNA. Lanes: 1 and 2, total RNase-resistant RNA; 3 and 4, RNA recovered from the most slowly migrating zone of ds PSTV RNA; 5 and 6, RNA recovered from the most rapidly migrating zone. Samples in lanes 1, 3, and 5 were not denatured before electrophoresis, while those in lanes 2, 4, and 6 were heated for 2 min at 100°C in the presence of 50% formamide. Equal autoradiographic exposures were used for PSTV (*A*) and cPSTV (*B*) analyses. Arrows, $PSTV_C$ (upper) and $PSTV_L$ (lower) markers.

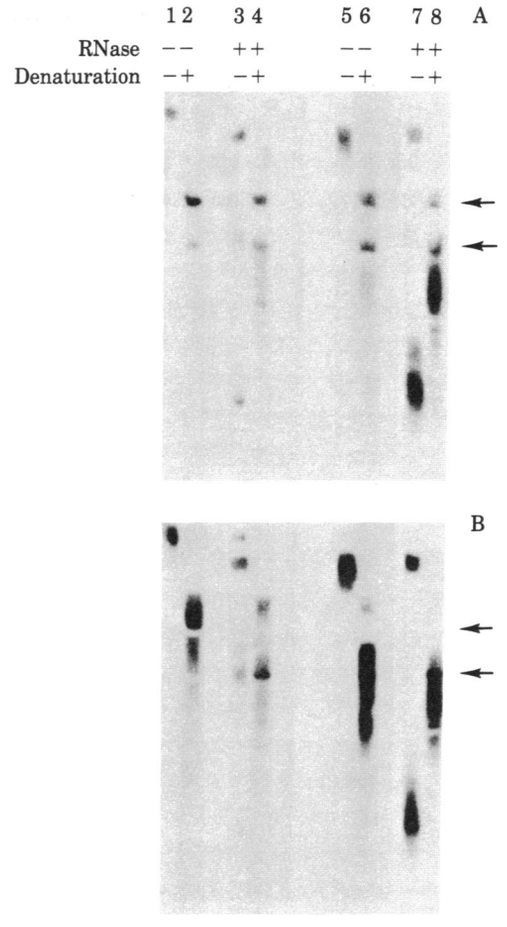

FIG. 3. Determination of sizes of the PSTV (*A*) and cPSTV (*B*) strands present in ds PSTV RNA. ds PSTV RNA was isolated from 2.4 mg of 2 M LiCl-soluble RNA by CF-11-cellulose chromatography and fractionated by preparative electrophoresis. One-half of each sample was incubated with pancreatic RNase and recovered by phenol extraction and ethanol precipitation (lanes 3, 4, 7, and 8); the other half was incubated in the absence of RNase (lanes 1, 2, 5, and 6). Samples were again divided into halves—one half was denatured before electrophoresis (lanes 2, 4, 6, and 8), while the other half was not (lanes 1, 3, 5, and 7). Each lane contains RNA equivalent to 300 μg of 2 M LiCl-soluble RNA. Autoradiographic exposure for cPSTV analysis was twice that for PSTV analysis. Arrows, $PSTV_C$ (upper) and $PSTV_L$ (lower) markers.

cPSTV strand decreased and a discrete band of $cPSTV_L$ appeared, a portion of the cPSTV in the more slowly migrating species was not affected by RNase digestion (compare lane 2 with lane 4 and lane 6 with lane 8).

Involvement of ds PSTV RNA in PSTV Replication. These ds PSTV RNA species have properties expected for intermediates in a RNA-directed RNA replication mechanism, but no *direct* evidence for their involvement in PSTV replication has been presented. Because unpublished data indicated that differential temperature treatment (21) of PSTV-infected tomato seedlings can produce an experimental system in which PSTV replication is at least partially synchronized, the relative amounts of PSTV and ds PSTV RNA at various times after the temperature increase were estimated by electrophoresis (Fig. 4).

While viroid replication was blocked by the low temperature, the upper part of the tomato seedlings contained only traces of PSTV and a small amount of ds PSTV RNA. Twenty-four hours after the shift to the permissive temperature (31°C), the concentration of PSTV had increased approximately 100-fold; a marked increase in the amount of ds PSTV RNA accompanied this increase in PSTV. The ds PSTV RNA/PSTV ratio was maintained over the next 74 hr, a period during which the PSTV concentration remained constant. Significant amounts of ss cPSTV were not observed.

DISCUSSION

The unusual secondary structure of plant viroids must be considered in studies involving nucleic acid hybridization techniques. The extensive intramolecular complementarity of PSTV has been shown to result in the hybridization of ^{125}I-labeled PSTV with unlabeled $PSTV_C$ and $PSTV_L$ under high salt/low temperature conditions (22). A combination of defined homogeneous hybridization probes and stringent hybridization conditions appears, however, to have prevented hybridization of cPSTV probes with PSTV and PSTV probes with cPSTV in our experiments.

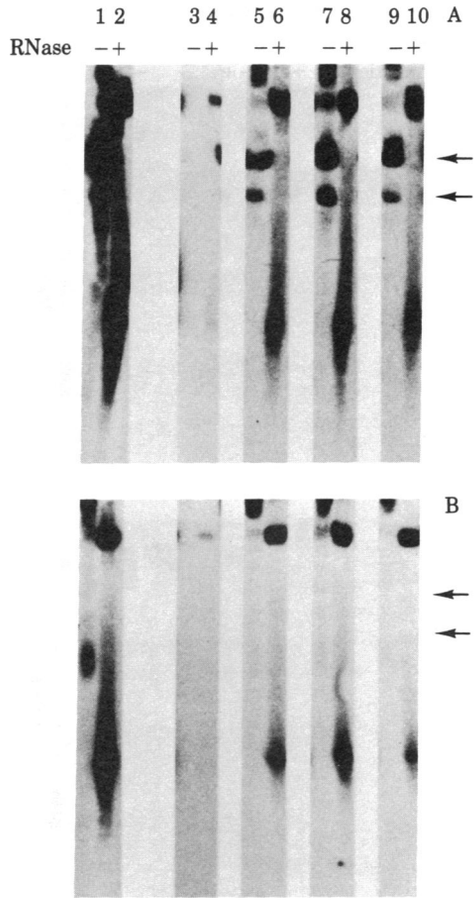

FIG. 4. Appearance of PSTV and ds PSTV RNAs during synchronized PSTV replication. Total RNA was prepared from the upper parts of tomato seedlings at the indicated times after returning the temperature to 31°C. Aliquots of these RNAs and total RNA prepared from unsynchronized PSTV-infected plants were incubated with pancreatic RNase and recovered by phenol extraction and ethanol precipitation (lanes 2, 4, 6, 8, and 10). Samples for PSTV analysis (*A*) contained either 5 μg of untreated RNA or the equivalent of 50 μg of RNase-resistant RNA; samples for cPSTV analysis (*B*) contained either 10 μg of untreated RNA or the equivalent of 50 μg of RNase-resistant RNA. Lanes: 1 and 2, total RNA from unsynchronized plants; 3 and 4, RNA from synchronized plants harvested before the temperature was returned to 31°C; 5–10, RNA from synchronized plants harvested 24 (lanes 5 and 6), 72 (lanes 7 and 8), and 98 (lanes 9 and 10) hr after the return to 31°C. The concentration of PSTV in the unsynchronized plants was ≈1000-fold greater than that in the synchronized plants before the temperature shift and ≈10-fold greater than that in the synchronized plants 24 hr after the temperature shift. Arrows, $PSTV_C$ (upper) and $PSTV_L$ (lower) markers.

The 300-nucleotide probe for PSTV and the 59-nucleotide probe for cPSTV are homogeneous DNAs of known sequence obtained by recombinant DNA techniques (9, 19). The procedures used in their preparation were specifically designed to produce cPSTV probes of maximum purity. Our low salt/high temperature hybridization conditions are much more stringent than those commonly used: the 55°C hybridization temperature is slightly higher than the calculated melting temperature for PSTV in 40% formamide/0.18 M NaCl (23, 24) and ≈10°C less than that calculated for DNA·RNA hybrids containing ≈50% G+C. Direct determination of the melting temperatures of our DNA·PSTV and DNA·cPSTV hybrids gave the expected values (≥80°C in 0.18 M NaCl; results not shown). No hybridization to RNA extracted from uninfected tissue was observed.

A schematic summary of our results and tentative structures for ds RNA intermediates of PSTV replication are shown in Fig. 5. Electrophoresis of RNA extracted from PSTV-infected tomato tissue under conditions that denature ss RNA but not ds RNA resolved at least five discrete zones of viroid-related RNA (Fig. 5 *Left*). Two zones contain ss RNAs, the circular and linear forms of PSTV and cPSTV. Only traces of ss $cPSTV_C$ and $cPSTV_L$ could be detected (Fig. 1), confirming our previous conclusion that most cPSTV is isolated as RNase-resistant duplexes also containing PSTV (9) and in accord with studies of plant RNA virus replication.

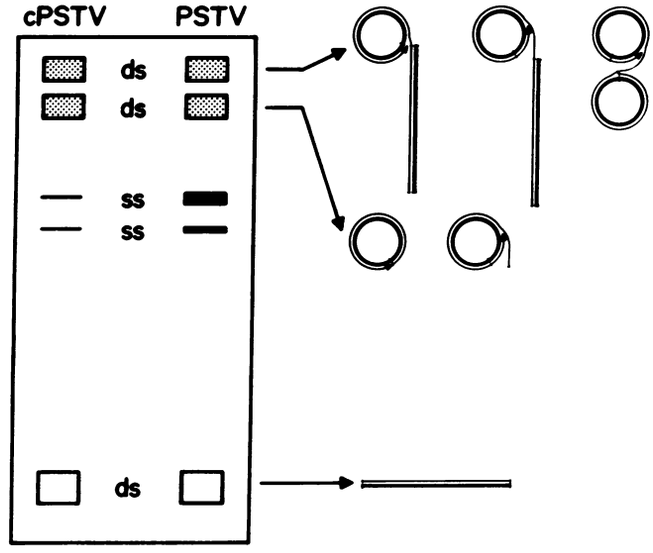

FIG. 5. Major viroid-related RNA species characterized and tentative structures for ds PSTV RNA. The mobility of each of the five PSTV-related zones (three containing ds PSTV RNA and two containing ss PSTV or cPSTV) has been calculated relative to that of $PSTV_C$. Shading indicates relative amounts present in a total RNA preparation. $PSTV_C$ was the most abundant species, while the $PSTV_L$·$cPSTV_L$ duplex is shown as an empty box to indicate that it was not detected unless RNA samples had been digested with RNase. [^{32}P]DNA probe specificities are indicated. Thick lines, Monomeric PSTV strands in the ds PSTV RNAs (both the $PSTV_C$ template and the $PSTV_C$ and $PSTV_L$ progeny); thin lines, linear cPSTV strands of various lengths.

At least three zones contain ds RNA, as shown by RNase treatment, and their structures have been analyzed. The most rapidly migrating ds RNA species, a linear duplex containing $PSTV_L$ and $cPSTV_L$, was not detected unless the RNA had been incubated *in vitro* with RNase. It is not, therefore, considered a potential intermediate in PSTV replication. Two more slowly migrating ds PSTV RNAs, however, possess several of the characteristics expected for such intermediates.

The more rapidly migrating of these two ds RNAs was quite resistant to digestion by RNase. When isolated after digestion by RNase, it contained $cPSTV_L$ and either $PSTV_C$ or $PSTV_L$ (Fig. 2). We conclude that two types of circular ds PSTV molecules are present in this zone: in some only the cPSTV strand is nicked, while in others both strands are nicked (at different positions). When isolated by CF-11-cellulose chromatography and preparative electrophoresis the more rapidly migrating ds PSTV RNA contains cPSTV strands somewhat longer than $cPSTV_L$ (Fig. 3, lane 6). The more slowly migrating ds PSTV RNA was much more sensitive to digestion by RNase than the more rapidly migrating one; it also contains cPSTV strands longer than unit length (Fig. 3, lane 2), ranging from a linear monomer to at least a dimer.

Neither of the two slowly migrating ds PSTV RNAs can be definitely classified as either a viroid RF or RI. The solubility of the more rapidly migrating ds PSTV RNA in 2 M LiCl and

its partial resistance to digestion by RNase resemble properties of viral RFs, but the presence of longer than unit-length cPSTV strands is unusual. The most slowly migrating ds PSTV RNA resembles a viral RI in its much greater sensitivity to digestion by RNase but, in contrast to viral RIs, it is soluble in 2 M LiCl.

Other investigators (7, 8, 22) have reported the existence of presumptive viroid RIs that are insoluble in 2 M LiCl. This apparent discrepancy may be explained by differences in the RNA isolation procedures used; 80–90% of both the RF and RI of tobacco mosaic virus can be specifically fragmented by high-speed homogenization during RNA isolation (25). This apparently specific fragmentation may be a consequence of "different functional states" of these intermediates, because RFs of three other viruses were not fragmented by homogenization (25). Double-stranded PSTV RNA, a duplex containing both circular and linear strands, may be extremely susceptible to shear during isolation.

Elements of a tentative model of PSTV replication that is consistent with our data are shown in Fig. 5 *Right*. The most unusual feature of the slowly migrating ds PSTV RNAs is the presence of cPSTV strands distinctly longer than unit length. Their formation can most readily be explained if one assumes that cPSTV is synthesized on a circular PSTV template and that this synthesis continues past the origin of replication leading to the synthesis of linear dimers and higher multimers of cPSTV. Such a scheme resembles, in some respects, the "rolling circle" model previously advanced to explain replication of certain viral RNAs (26).

Our model further posits that monomeric $PSTV_L$ synthesized from the multimeric cPSTV template is circularized while still complexed to the multimeric cPSTV. Multimeric PSTV molecules have not been detected, but their synthesis on a multimeric cPSTV template can easily be visualized. A comprehensive mechanism for PSTV replication must also accommodate the synthesis of small amounts of ss $cPSTV_C$ and $cPSTV_L$ monomers.

In this view, the more slowly migrating ds PSTV RNA zones would contain dimers and higher multimers of cPSTV, whereas the more rapidly migrating ds PSTV RNA zone would contain circular ds monomers with ss cPSTV tails of various lengths. The model accounts for the formation of more rapidly migrating circular ds RNA as well as linear $PSTV_L$·$cPSTV_L$ duplexes during digestion of the most slowly migrating ds PSTV RNA by RNase. Although the most slowly migrating ds PSTV RNA zone characterized appears to contain a dimeric cPSTV strand, larger RF and RI molecules would have the same basic structure and strand sensitivities to digestion by RNase.

Multimeric ds RNAs have been detected during replication of at least one RNA plant virus. The molecular weight of the complementary RNA in the satellite of tobacco ringspot virus RF is up to 20 times that of the satellite RNA; pancreatic RNase digestion of these multimeric RFs releases smaller ds RNAs that contain biologically active satellite RNA (27).

In summary, we seem to have isolated, as ds PSTV RNA, fragments of a large PSTV RI complex containing a multimeric linear cPSTV strand complexed with monomeric circular and linear PSTV progeny. Although isolated ds RNA may be an artifact of the RNA extraction procedure (28), the *in vivo* precursor to our ds PSTV RNAs seems to be involved in PSTV replication. Synchronous synthesis of ss PSTV is accompanied by a simultaneous marked increase in ds PSTV RNA (Fig. 4). Therefore, further characterization of ds PSTV RNA isolated by methods designed to minimize fragmentation of these unusual RNAs should help to identify the detailed mechanism of viroid replication.

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1. Diener, T. O. (1979) *Science* **205**, 859–866.
2. Gross, H. J. & Riesner, D. (1980) *Angew. Chem. Int. Ed. Engl.* **19**, 231–243.
3. Hadidi, A., Jones, D. M., Gillespie, D. H., Wong-Staal, F. & Diener, T. O. (1976) *Proc. Natl. Acad. Sci. USA* **73**, 2453–2457.
4. Zaitlin, M., Niblett, C. L., Dickson, E. & Goldberg, R. B. (1980) *Virology* **104**, 1–9.
5. Branch, A. D. & Dickson, E. (1980) *Virology* **104**, 10–26.
6. Hadidi, A., Cress, D. E. & Diener, T. O. (1981) *Proc. Natl. Acad. Sci. USA* **78**, 6932–6935.
7. Grill, L. K. & Semancik, J. S. (1978) *Proc. Natl. Acad. Sci. USA* **75**, 896–900.
8. Grill, L. K., Negruk, V. I. & Semancik, J. S. (1980) *Virology* **107**, 24–33.
9. Owens, R. A. & Cress, D. E. (1980) *Proc. Natl. Acad. Sci. USA* **77**, 5302–5306.
10. Rackwitz, H. R., Rohde, W. & Sänger, H. L. (1981) *Nature (London)* **291**, 297–301.
11. Siegel, A. & Hariharasubramanian, V. (1974) in *Comprehensive Virology*, eds. Fraenkel-Conrat, H. & Wagner, R. R. (Plenum, New York), Vol. 2, pp. 61–108.
12. Diener, T. O., Hadidi, A. & Owens, R. A. (1977) in *Methods in Virology*, eds. Maramorosch, K. & Koprowski, H. (Academic, New York), Vol. 6, pp. 185–217.
13. Franklin, R. M. (1966) *Proc. Natl. Acad. Sci. USA* **55**, 1504–1511.
14. Engelhardt, D. L. (1972) *J. Virol.* **9**, 903–908.
15. Maxam, A. M. & Gilbert, W. (1980) *Methods Enzymol.* **65**, 499–560.
16. Wahl, G. M., Stern, M. & Stark, G. R. (1979) *Proc. Natl. Acad. Sci. USA* **76**, 3683–3687.
17. Alwine, J. C., Kemp, D. J., Parker, B. A., Reiser, J., Renart, J., Stark, G. R. & Wahl, G. M. (1980) *Methods Enzymol.* **68**, 220–242.
18. Stellwag, E. J. & Dahlberg, A. E. (1980) *Nucleic Acids Res.* **8**, 299–317.
19. Cress, D. E. & Owens, R. A. (1981) *DNA* **1**, 90 (abstr.).
20. Kaper, J. M. & Diaz-Ruiz, J. R. (1977) *Virology* **80**, 214–217.
21. Dawson, W. O. & Schlegel, D. E. (1973) *Virology* **53**, 476–478.
22. Rohde, W. & Sänger, H. L. (1981) *Biosci. Rep.* **1**, 327–336.
23. Langowski, J., Henco, K., Riesner, D. & Sänger, H. L. (1978) *Nucleic Acids Res.* **5**, 1589–1610.
24. Palukaitis, P. & Symons, R. H. (1979) *Virology* **98**, 238–245.
25. Dawson, W. O., German, T. L. & Schlegel, D. E. (1976) *J. Gen. Virol.* **32**, 205–215.
26. Brown, F. & Martin, S. J. (1965) *Nature (London)* **208**, 861–863.
27. Schneider, I. R. & Thompson, S. M. (1977) *Virology* **78**, 453–462.
28. Garnier, M., Mamoun, R. & Bové, J. M. (1980) *Virology* **104**, 357–374.